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PATENT APPLICATION

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of

Docket No: Q62106

Shotaro YAMAGUCHI

Appln. No.: 09/727,769

Group Art Unit: 1652

Confirmation No.: 5479

Examiner: Manjunath N. Rao

Filed: December 4, 2000

For: NOVEL PROTEIN-DEAMIDATING ENZYME, MICROORGANISM PRODUCING THE SAME, GENE ENCODING THE SAME, PRODUCTION PROCESS THEREFOR, AND USE THEREOF

DECLARATION UNDER 37 C.F.R. § 1.132

(Re: Enablement)

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

I, Shotaro YAMAGUCHI, a citizen of Japan, hereby declare and state:

I graduated from Graduate School, Faculty of Agriculture of Kyoto University with a Master's Degree in the field of Food Science and Technology in March of 1984.

In April of 1984, I was employed by Amano Pharmaceutical Co., Ltd. (Amano Pharmaceutical Co. has since changed its name to Amano Enzyme Co., Ltd.).

In January of 1992, I received a Ph.D. from Kyoto University.

From February of 1998 until March of 2001, I was resident at the Institute of Food Research (Colney, Norwich NR4 7US, U.K.) as a visiting scientist.

Since April of 2001 I have been working at Amano Enzyme Co., Ltd., and since 1984, I hav been engaged in research and development of enzymes, and in the study f their applications in the field of foods.

I am considered to be an expert in the field of the present invention. I am further fully knowledgeable of the disclosure of the present application (U.S. Patent Application No. 09/727,769) and the materials contained therein.

I am aware of the Advisory Action mailed August 29, 2003, in the above-identified application, wherein the examiner maintains the rejection of claims 26, 29, 30, 33, 34, and 37 under 35 U.S.C. § 112, first paragraph; asserting that the specification is not enabling for any polynucleotide which is 80% homologous to polynucleotides encoding the polypeptide with SEQ ID NO:6, or 80% homologous to SEQ ID NO:5, or for polynucleotides which hybridize with such polynucleotides under stringent conditions.

The examiner contends that the specification is not enabling for the use of deaminating enzymes from any source, or for the use of recombinant or variant deamidases. Furthermore, the Examiner asserts that while mutagenesis and recombinant techniques are known in the art, producing variant deamidases requires that one of ordinary skill in the art be provided with guidance for the selection of which, of the infinite number of variants, have the claimed property. The examiner asserts that without such guidance one of ordinary skill would be left with extensive and undue experimentation.

In my opinion, the examiner's position is incorrect.

Specifically, the examiner is incorrect in asserting that one of ordinary skill in the art must be provided with guidance for the selection of which, of the infinite numbers of variants, have the claimed property. This is because the selection occurs by ruling out all

sequences that do not have sufficient homology and/or that do not hybridize with the polynucleotide which has the nucleotide sequence of SEQ ID NO:5. This selection step narrows the remaining candidates to nucleotide sequences that are generally expected to encode an active enzyme. As evidence that my opinion is sound, note that the nucleotide sequence of the deamidation enzyme of the present application (U.S. Application No: 09/727,769), related to *Chryseobacterium* sp. 9670, and that of related application No: 09/793,495, re *Chryseobacterium gleum*, are about 76% homologous. This is compelling evidence that one would expect nucleotide sequences having 80% homology to a nucleotide sequence encoding an active enzyme, to also encode active enzymes.

Furthermore, the hybridization method identifies DNA having nucleotide homology with the template DNA by utilizing the ability to form a double strand. Because this method uses the sequence actually obtained, SEQ ID NO:5, one skilled in the art would expect that sequences having a high homology can be obtained under stringent conditions.

This selection step, having narrowed the sequences on the basis of homology, either directly or via hybridization, provides a pool of candidate polynucleotides that are expected to encode active enzymes. Screening this pool of polynucleotides for activity would not be undue experimentation, but rather routine.

I declare further that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of

the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Date: Oct. 23, 2003

Shotaro YAMAGUCHI

PATENT APPLICATION

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of

Docket No: Q62106

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Appln. No.: 09/727,769

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For: NOVEL PROTEIN-DEAMIDATING ENZYME, MICROORGANISM PRODUCING THE SAME, GENE ENCODING THE SAME, PRODUCTION PROCESS THEREFOR, AND USE THEREOF

DECLARATION UNDER 37 C.F.R. § 1.132

(Re: Written Description)

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

I, Shotaro YAMAGUCHI, a citizen of Japan, hereby declare and state:

I gradnated from Graduate School, Faculty of Agriculture of Kyoto University with a Master's Degree in the field of Food Science and Technology in March of 1984.

In April of 1984, I was employed by Amano Pharmaceutical Co., Ltd. (Amano Pharmaceutical Co. has since changed its name to Amano Enzyme Co., Ltd.).

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From February of 1998 until March of 2001, I was resident at the Institute of Food Research (Colney, Norwich NR4 7US, U.K.) as a visiting scientist. Since April of 2001 I have been working at Amano Enzyme Co., Ltd., and since 1984, I have been engaged in research and development of enzymes, and in the study of their applications in the field of foods.

I am considered to be an expert in the field of the present invention. I am further fully knowledgeable of the disclosure of the present application (U.S. Patent Application No. 09/727,769) and the materials contained therein.

I am aware of the Advisory Action mailed August 29, 2003, in the above identified application, wherein the Examiner maintains the rejection of claims 26, 30, and 34 under 35 U.S.C. § 112, first paragraph; asserting that the claims do not comply with the written description requirement. Specifically, the Examiner contends that Applicants have only shown the presence of the enzyme in one microorganism, and have not shown that the enzyme is produced by a large number of microorganisms.

On the contrary, the present inventors described five strains of the genus *Chryseobacterium* that can produce a protein-deamidating enzyme with the activity shown in Example 4 of the specification (pages 57 and 58). These microorganisms include one obtained by screening microorganisms of the natural world and four obtained from the type culture. Although six microorganisms were known at the time of filing this application (Applied and Environmental Microbiology, Aug. 2000, p. 3337-3343), only four of them were available. Since all microorganisms tested from the genus *Chryseobacterium* were shown by Applicants to produce a protein-deamidating enzyme, one skilled in the art would consider it highly probable that all microorganisms belonging to the genus *Chryseobacterium* also produce the protein-deamidating enzyme.

I declare further that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are

punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Date: Oct. 23, 2003

Shotaro YAMAGUCHI

APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Aug. 2000, p. 3337–3343
0099-2240/00/$04.00+0

Vol. 66, No. 8

A Novel Protein-Deamidating Enzyme from *Chryseobacterium proteolyticum* sp. nov., a Newly Isolated Bacterium from Soil

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Received 14 March 2000/Accepted 30 May 2000

A novel protein-deamidating enzyme, which has potential for industrial applications, was purified from the culture supernatant of *Chryseobacterium proteolyticum* strain 9670^T isolated from rice field soil in Tsukuba, Japan. The deamidating activities on carboxybenzoxy (Cbz)-Gln-Gly and caseins and protease activity were produced synchronously by the isolate. Both deamidating activities were eluted as identical peaks separated from several proteases by phenyl-Sepharose chromatography of the culture supernatant. The enzyme catalyzed the deamidation of native caseins with no protease and transglutaminase activities. Phenotypic characterization and DNA analyses of the isolate were performed to determine its taxonomy. Physiological and biochemical characteristics, 16S rRNA gene sequence analysis, and DNA-DNA relatedness data indicated that the isolate should be placed as a new species belonging to the genus *Chryseobacterium*. The isolate showed no growth on MacConkey agar and produced acid from sucrose. The levels of DNA-DNA relatedness between the isolate and other related strains were less than 17%. The name *Chryseobacterium proteolyticum* is proposed for the new species; strain 9670 is the type strain (=FERM P-17664).

An enzyme catalyzing deamidation of proteins has a great potential for industrial applications. Deamidation of proteins can improve protein functionalities such as solubility, emulsification, and foaming and gelation properties, which are desired properties in some food proteins. Most plant proteins have poor solubility and functionality under mild acidic conditions, which are the pH ranges of most food systems, resulting in their limited use in foods. Because the contents of glutamine residue in plant proteins are generally high, deamidation of such proteins is one of the most promising ways to expand their uses and to improve their functionalities. Many studies of the chemical (mild acid or alkaline treatment) or physical (dry heat treatment) deamidation of food proteins had reported and demonstrated the effectiveness of deamidation for improvement of protein functionalities (see reference 25 for review). To avoid unfavorable side effects brought about by nonenzymatic treatments—for example, concomitant peptide bond cleavage, off-flavor formation, and amino acid racemization—enzymatic deamidations of proteins have been examined (see reference 10 for review). Protease (16), transglutaminase (23), and peptidoglutaminase (9) were used for this purpose. None of their primary reactions were deamidations, or the enzymic substrates were peptides rather than proteins. Besides the improvement in protein functionalities, protein-deamidating enzymes could be used for many applications, including protein structure analysis.

In 1971, Kikuchi et al. (17) found an enzyme, peptidoglutaminase, from *Bacillus circulans* that deamidates the peptide-bound glutamines. This enzyme was not active on high-molecular-weight peptides, i.e., proteins such as caseins, unless the proteins were hydrolyzed to short peptides (17). In plants, the possible presence of protein deamidase in germinating wheat grains was reported, but the enzyme has not yet been fully purified and characterized (31).

To obtain a protein-deamidating enzyme of microbial origin, we have screened microorganisms from soils and successfully isolated a bacterium that produces the target enzyme. The enzyme deamidated native caseins without protease and transglutaminase activity. In the present study, we report the discovery of a novel protein-deamidating enzyme from a bacterium and the taxonomic determination of the isolate. The latter led to the proposal of a new species within the genus of *Chryseobacterium*.

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MATERIALS AND METHODS

Bacterial strains. Two strains, 9670^T and 9671, isolated as described below were used. They were maintained on nutrient agar at 4°C. Type strains of *Chryseobacterium gleum* JCM 2410^T (ATCC 35910^T), *Chryseobacterium indologenes* IFO 14944^T (ATCC 29897^T), *Chryseobacterium balustinum* IFO15053^T (ATCC 33487^T), *Chryseobacterium meningosepticum* IFO 12535^T (ATCC 13253^T), *Empedobacter brevis* IFO 14943^T (NCTC 11099^T), and *Myroides odoratus* IFO 14945^T (ATCC 4651^T) were used as reference strains for DNA-DNA hybridization studies.

Isolation of strains. Water suspensions of 320 soil samples, collected from natural environments, such as grasslands, gardens, crop fields, livestock farms, riversides, forests, and dumping grounds in Tsukuba City, Japan, were inoculated into A medium consisting of 0.1% carboxybenzoxy (Cbz)-Gln-Gly (Peptide Laboratory, Osaka, Japan), 0.5% glucose, 0.02% KH_2PO_4, 0.02% $MgSO_4 \cdot 7H_2O$, 0.01% NaCl, 0.002% $CaCl_2$, 0.0002% $FeSO_4 \cdot 7H_2O$, 0.0005% $NaMO_4 \cdot 2H_2O$, 0.0005% $NaWO_4 \cdot 4H_2O$, 0.0005% $MnSO_4 \cdot 4H_2O$, and 0.01% $CuSO_4 \cdot 5H_2O$ (pH 8.0, adjusted with 6N NaOH). The cultures were incubated aerobically at 30°C for 6 days. Fresh A medium was inoculated with a portion of the above cultures and incubated at 30°C for 3 days. Bacterial and fungal strains were isolated by plating or streaking a portion of the second culture onto Luria-Bertani agar (Oxoid, Basingstoke, United Kingdom) for bacteria or potato-dextrose agar (Difco Laboratories, Detroit, Mich.) for fungi. Isolated strains were inoculated onto A medium containing 1.5% agar. Strains grown on the plates were collected and then inoculated into B medium consisting of 0.5% lactose, 1.0% peptone, 0.17% $Na_2HPO_4 \cdot H_2O$, 0.025% KH_2PO_4, 0.025% $MgSO_4 \cdot 7H_2O$, and 0.005% $FeSO_4 \cdot 7H_2O$ (pH 7.2, adjusted with 6N NaOH). The cultures were incubated aerobically at 30°C for a period of from 2 to 7 days. Culture supernatants were subjected to enzyme assays. Two strains showing higher protein-deamidating activity were selected and purified by repeated streaking on the nutrient agar medium.

Enzyme assays. For deamidating activity, 100 μl of substrate solution containing 10 mM Cbz-Gln-Gly or 1.0% caseins (Hammersten, Merk, Poole, United Kingdom), 175.6 mM sodium phosphate buffer (pH 6.5), and 10 μl of enzyme solution were mixed and then incubated at 37°C for 60 min. The reaction was stopped by the addition of 100 μl of 12% trichloroacetic acid. For blank assays, enzyme solution was added after addition of trichloroacetic acid. After centrifugation at 18,000 × *g* for 5 min, released ammonia in the supernatant was determined by an NADH-glutamate dehydrogenase method (21) with an am-

monia determination kit according to the manufacturer's instructions (Boehringer-Mannheim/Roche Diagnostics, Lewes, United Kingdom). One unit of enzyme was defined as the amount that released 1 μmol of ammonia per min under the above conditions. Ammonia was also determined by a phenol method for screening study. In this case, 10 μl of the supernatant was mixed with 100 μl of 1.0% phenol–0.005% sodium nitroprusside, and then 100 μl of 0.5% NaOH–0.6% NaOCl was added. After 60 min, the A_{630} of the mixture was measured. For protease activity, the A_{280} was measured in the supernatant from the above deamidating activity assay when casein was used as a substrate. One unit of protease activity was defined as the amount that caused an increase of 1 optical density unit at 280 nm (OD_{280}) per 60 min under the above conditions. Transglutaminase activity was assayed by a hydroxamate method according to Folk and Chung (5).

Partial purification of protein-deamidating enzyme. A preculture of strain 9670^T in B medium grown at 25°C overnight was inoculated into the same medium at a 1.0% concentration of preculture. The culture was incubated at 25°C with reciprocal shaking at 145 rpm for 48 h for a culture profile study or 24 h for enzyme purification. The culture broth was centrifuged at 22,200 × *g* for 15 min at 4°C. After addition of 2 mM EDTA, the supernatant was concentrated eightfold by ultrafiltration with a hollow-fiber-type membrane (AIP1010, MW 6000 cut; Asahi Chemical Industry, Tokyo, Japan). After dialysis of the concentrate against 2.0 M NaCl in 10 mM sodium phosphate (pH 6.5), the dialysate was centrifuged at 2,200 × *g* for 10 min at 4°C and filtered through a 0.45-μm-pore-diameter membrane in order to remove the insoluble materials. The resultant filtrate was applied to a phenyl-Sepharose High Performance Hiload 16/10 column (Amersham Pharmacia Biotech, Uppsala, Sweden) prequilibrated with 2.0 M NaCl in 10 mM sodium phosphate buffer (pH 6.5). The column was washed with two column volumes of 2.0 M NaCl in 10 mM sodium phosphate (pH 6.5), and absorbed proteins were eluted by an NaCl gradient from 2.0 to 0 M in 88 ml of 10 mM sodium phosphate buffer (pH 6.5). The elution was followed with 10 mM sodium phosphate buffer (pH 6.5). The chromatography was carried out with a fast-performance liquid chromatography (FPLC) system (Amersham Pharmacia Biotech) with all flow rates at 1.0 ml/min.

SDS-PAGE. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed by the method of Laemmli (20) using a 10 to 20% polyacrylamide gradient gel (Multigel; Daiichi Pure Chemicals, Tokyo, Japan). Proteins were silver stained with a kit from Wako Pure Chemicals, Osaka, Japan. Molecular weight markers were obtained from Daiichi Pure Chemicals.

Determination of phenotypic characteristics. Morphological and cultural characteristics were observed on nutrient agar (Eiken, Tokyo, Japan) and Trypto-Soy agar (Eiken). The pH range for growth was determined in nutrient broth (Difco) filtered through a 0.45-μm-pore-diameter membrane after adjustment to various pHs with HCl or NaOH. The cells grown on Trypto-Soy agar at 30°C were recorded by scanning electron microscopy. Physiological and biochemical characteristics were determined according to reference 1 and Yabuuchi et al. (35). Hydrolyses of DNA, gelatin, and esculin were examined with DNA test agar (Difco), heart infusion broth (Difco) containing 12% gelatin, and a medium consisting of 1% Bacto Peptone (Difco), 0.5% NaCl, 0.05% ferric citrate, and 0.1% esculin, respectively. Indole production was tested by using 2% tryptone (Difco) broth and Kovacs reagent (7). Urease activities, nitrate reduction, and malonate utilization were examined by using Christensen urease test agar (Eiken), nutrient broth (Eiken) containing 0.1% potassium nitrate, and malonate-phenylalanine medium (Kyokuto, Tokyo, Japan), respectively. MacConkey agar was from Eiken. Acid and gas formations from sugar were examined by using ammonia salt-sugar medium [0.1% $(NH_4)_2HPO_4$, 0.02% KCl, 0.02% $MgSO_4 \cdot 7H_2O$, 0.02% yeast extract, 1.5% agar, 0.002% bromcresol purple] and O-F basal medium (Difco) containing 1% of each sugar. Flexirubin-type pigment was detected according to the method of Yabuuchi et al. (35).

16S rRNA gene sequencing and analysis. Isolation of genomic DNA, PCR-mediated amplification of 16S rRNA gene, and purification and sequencing of the PCR product were performed according to the method of Shida et al. (28). Oligonucleotide primers 5′-CTGGGATCCATTTACTCGAGAGTTTGATCCTGGCTCAG-3′ (5′ end of the 16S rRNA gene) and 5′-GGTTCCCCTAAGCTTACCTTGTTACGACTTC-3′ (3′ end of the 16S rRNA gene) were used for PCR amplification of the 16S rRNA gene as described by Shida et al. (27). The amplified 16S gene was purified with a QIAquick spin PCR purification kit (Qiagen GmbH, Hilden, Germany) and then used as a sequencing template. Seven sequencing primers were used as described by Fox et al. (6). The sequence determined was compared with 16S rRNA gene sequences obtained from the EMBL, GenBank, and DDBJ databases. Multiple alignment of sequences, calculation of nucleotide substitution rates (K_{nuc} values) (18), construction of a neighbor-joining phylogenetic tree (26), and a bootstrap analysis with 1,000 replicates for evaluation of phylogenetic tree topology (4) were performed with the CLUSTAL W version 1.5 program (30).

DNA base composition and DNA-DNA hybridization. The G + C content of the DNA was determined by the method of Tamaoka and Komagata (29). Levels of DNA-DNA relatedness were determined fluorometrically by the method of Esaki et al. (3) with photobiotin-labeled DNA probes and microplates.

Nucleotide sequence accession number. The nucleotide sequence data of the 16S rRNA gene of strain 9670^T have been deposited in the DDBJ, EMBL, and GenBank nucleotide sequence databases under accession no. AB039830.

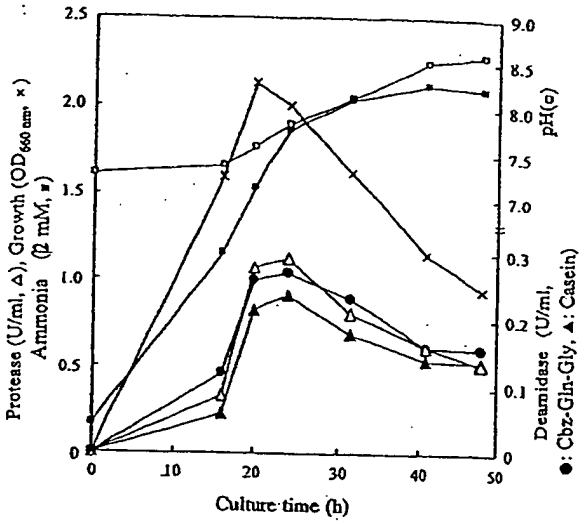

FIG. 1. Culture profile of strain 9670^T. Culture conditions were described in Materials and Methods. Cell growth (×) in liquid culture was monitored by measuring the OD_{660}. Ammonia (■) was determined by the NADH-glutamate dehydrogenase method. Deamidating activities were determined on Cbz-Gln-Gly (●) and casein (▲). Protease activity (△) was also determined.

RESULTS

Isolation of bacterial strains. Repeated liquid cultures and subsequent plate culture using Cbz-Gln-Gly as the sole nitrogen source were used to enrich for protein-deamidating enzyme producers from soils. From 320 soil samples, 150 bacteria and 294 fungi were isolated and examined for protein-deamidating enzyme productivity in their culture supernatants. Among positive isolates, two bacteria showed significantly higher activities of deamidation of both Z-Gln-Gly and caseins. These isolates, designated as strains 9670^T and 9671, originated from soils of a rice field and the bank of a brook, respectively, and were used for the following studies.

Culture profile of the isolates. Figure 1 shows the culture profile of strain 9670^T. A similar profile was obtained for strain 9671. At the late exponential growth phase (16-h culture), deamidating activities on both Cbz-Gln-Gly and caseins began to be produced significantly and simultaneously. Protease activity was also produced, accompanied by the deamidating activities. The pH of the culture broth began to rise at the same time with increasing ammonia produced, which might be released from peptone contained in the medium by the deamidating activities. Maximum deamidating activities were observed at 24 h of culture with 0.258 U/ml on Cbz-Gln-Gly and 0.228 U/ml on caseins. The enzyme productivities of strain 9671 were lower than those of strain 9670^T by ca. 30%.

Partial purification of the protein-deamidating enzyme. Although ammonia-releasing activity from caseins was observed in the culture supernatants of the isolate, it was necessary to confirm whether the enzyme deamidated high-molecular-weight peptides, i.e., proteins, or merely deamidated short peptides produced by the protease activity. For this purpose, we tried to purify the deamidating activity from the culture supernatant of strain 9670^T. After trials of several kinds of chromatography and conditions, including ion-exchange chromatography, gel filtration, and chromatofocusing, it was found that hydrophobic chromatography on a phenyl-Sepharose column successfully resulted in the separation of deamidating

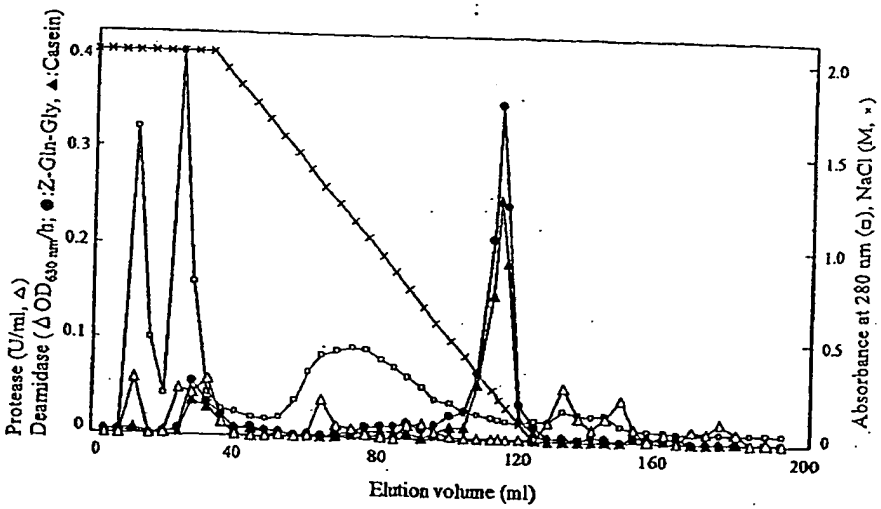

FIG. 2. Phenyl-Sepharose chromatography of the culture supernatant of strain 9670^T. Eluted proteins (□) were monitored by the OD_{280}. Both deamidating activities on Cbz-Gln-Gly (●) and casein (▲) were determined by the phenol method and expressed as the OD_{630}. Protease activity (△) was also determined.

activities from protease activities. The ultrafiltration and dialysis procedure described in Materials and Methods had also contributed to the removal of most of the protease activity (ca. 98%). At around 0.2 M NaCl in its gradient (elution volume of 113 ml), both deamidating activities on caseins and Cbz-Gln-Gly were eluted as identical peaks separated from several protease peaks (Fig. 2). A minor peak of deamidating activities was observed in the unabsorbed fraction (elution volume of 25 ml) with crossover by protease peaks. The main fraction for the deamidating enzyme at an elution volume of 113 ml was used in the following study. Protease activity in this fraction was less than the level of the minimal detection limit (<0.003 U/ml). Analysis by SDS-PAGE indicated this fraction contained a main protein band of 20 kDa with several minor protein bands.

Evidence of protein-deamidating enzyme. To confirm that the enzyme can deamidate high-molecular-weight proteins, caseins were incubated with the above enzyme fraction for 16.5 h at 37°C, and the products were subjected to SDS-PAGE. As shown in Fig. 3, casein treated with the deamidating enzyme fraction was scarcely degraded (lane 4), compared to the control casein treated without enzyme (lane 2), whereas casein was completely hydrolyzed by the culture supernatant which contains proteolytic activities besides the deamidating activity (lane 3). Ammonia released in these reaction mixtures was also determined and found to be at concentrations of 7.12, 0.02, and 8.04 mM in the reaction mixture with the deamidating enzyme, control casein mixture, and the reaction mixture with the culture supernatant, respectively. Provided an average molar content of amido-containing amino acid residues, Gln and Asn, in caseins of 26.8 mol/mol of protein and assuming an average molecular weight of casein of 23,261, which were calculated based on the numbers of both amino acids in four casein components (α_{S1}-, α_{S2}-, β-, and γ-caseins) and the relative content of each component in the casein preparation (34), the deamidation degree (millimolar ammonia released/millimolar total amido content in the substrate casein] × 100) was estimated as 62.0% for the reaction product by the deamidating enzyme fraction. These results imply that the casein treated by the deamidating enzyme fraction was deamidated in a high-molecular-weight state, not in a small-peptide state (i.e., not after degradation). It can be concluded, therefore, that the deamidating enzyme fractionated from the culture supernatant of strain 9670^T can deamidate high-molecular-weight proteins (caseins).

Slow migrations of protein bands in the casein treated with the deamidating enzyme fraction were observed (Fig. 3, lane 4 as compared to lane 2). Such slow migration on SDS-PAGE was reported for chemically deamidated gluten (2). This phenomenon might be considered to be caused by the increased negative charge in the deamidated protein, which should decrease the affinity between the protein and the negatively charged SDS molecule due to an electrostatic repulsion and

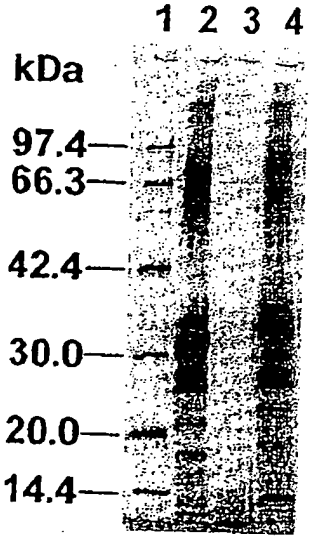

FIG. 3. SDS-PAGE of the caseins treated by the protein-deamidating enzyme fraction. Reaction conditions were the same as those in the casein-deamidating activity assay described in Materials and Methods, except for the reaction time (16.5 h). Lanes: 1, molecular mass markers; 2, control casein incubated with water (without enzyme); 3, reaction product treated by the culture supernatant; 4, reaction product treated by the protein-deamidating enzyme fraction.

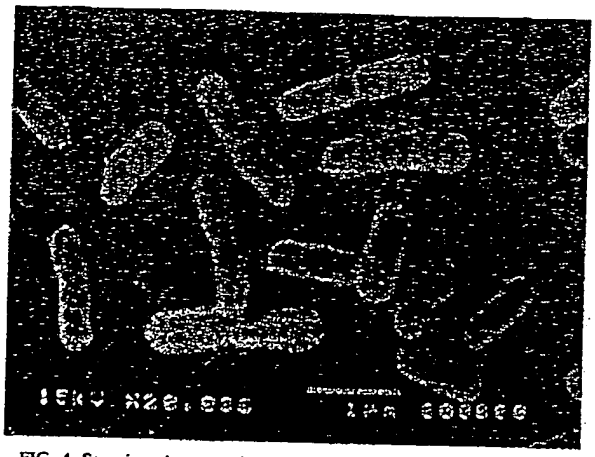

FIG. 4. Scanning electron micrograph of strain 9670^T cells from 24 h of culture. Bar, 1 μm.

then decrease the total negative charge of the protein-SDS complex.

The deamidating enzyme fraction had no transglutaminase activity, as measured by hydroxamate formation between Cbz-Gln-Gly and hydroxylamine, which is a common characteristic of transglutaminase. Furthermore, no formation of higher-molecular-weight, cross-linking products was observed in the reaction products of caseins by the deamidating enzyme fraction, as judged by SDS-PAGE (Fig. 3, lane 4 compared to lane 2). Casein is one of the best substrates for transglutaminase-catalyzed protein cross-linking, and the cross-linked products of casein can be easily detected by SDS-PAGE. The deamidating enzyme from strain 9670^T therefore could be distinguished from transglutaminase.

Phenotypic characteristics. Strains 9670^T and 9671 showed the same morphological and physiological characteristics. They were rod-shaped, nonmotile, and nonsporing. Gram staining was negative. The cells were 0.4 to 0.5 μm wide and 0.8 to 2.0 μm long (Fig. 4). They were aerobic and positive for oxidase and catalase, producing an insoluble yellow or orange pigment, which turned red with 3% KOH and returned to orange by neutralization, indicating a flexirubin type of pigment.

Phenotypic characterization of the isolates indicated that they were included in the genus *Chryseobacterium*, which belongs to the family *Flavobacteriaceae*. Differential characteristics were reported among seven genera of *Flavobacteriaceae*, including the genera *Chryseobacterium*, *Flavobacterium*, *Empedobacter*, *Weeksella*, *Bergeyella*, *Riemerella* (33), and *Myroides* (32). Except for the acid-forming property from sucrose, all other properties of the isolates matched those of *Chryseobacterium*. In the genus *Chryseobacterium*, six species (*C. gleum*, *C. indologenes*, *C. balustinum*, *C. indoltheticum*, *C. meningosepticum*, and *C. scophthalmum*) are recognized at present. Besides acid formation from sucrose, the new isolates were distinguished from these six existing species: acid formation from mannitol and growth on MacConkey agar for *C. gleum*; malonate utilization for *C. indologenes*, G+C content, acid formation from mannitol, growth at 36 to 37°C, and growth on MacConkey agar for *C. balustinum*; G+C content, acid formation from mannitol, and growth on MacConkey agar for *C. indoltheticum*; growth on MacConkey agar for *C. meningosepticum*; and acid formation from glucose and mannitol, growth at 36 to 37°C, urease activity, and indole production for *C. scophthalmum* (Table 1). These results indicated that the new isolates should be placed as a new species in the genus *Chryseobacterium*.

16S rRNA gene sequence and phylogenetic analysis. The determined 16S rRNA sequence of strain 9670^T showed higher similarities to those of a group consisting of several *Chryseobacterium* strains with 96.0, 95.9, 95.1, and 94.9% similarity to *C. gleum*, *C. indologenes*, *C. balustinum*, and *C. indoltheticum*, respectively. A recent published sequence of the 16S rRNA gene from *Chryseobacterium* sp. (22) showed 95.1% similarity to that of the strain 9670^T. A second group consisted

TABLE 1. Characteristics differentiating strains 9670^T and 9671 from existing *Chryseobacterium* species

Characteristic	Test result for[a]:						
	9670^T and 9671[b]	*C. gleum* (n = 12)[c]	*C. indologenes* (n = 13)[d]	*C. balustinum* (n = 1)[c]	*C. indoltheticum* (n = 1)[c]	*C. meningosepticum* (n = 49)[e]	*C. scophthalmum* (n = 7)[c]
G + C content (%)[f]	37.1 (n = 2)	37.6 ± 1.0 (n = 6)	37.7 ± 0.3 (n = 3)	33.1 (n = 1)	33.8 (n = 1)	37.0 ± 0.5 (n = 8)	34.2 ± 0.4 (n = 7)
Acid production from:							
Glucose	+	+	+	+	+	+	–
Sucrose	+	–	–	–	–	–	–
Mannitol	+	–	4/13[g]	–	–	31/49	–
Growth at 36–37°C	+	+	+	–	+	+	–
Growth on MacConkey agar	–	+	V[h]	+	+	+	–
Nitrate reduction	–	7/12	V	+	–	–	–
Urease activity	–	7/12	–	–	–	18/49	+
Indole production	+	+	+	+	+	24/49	–
Malonate utilization	–	NA[i]	+	NA	NA	NA	NA

[a] n is the number of strains tested. +, all strains tested positive; –, all strains tested negative.
[b] Results for strains 9670^T and 9671 were from this study.
[c] Data from Holmes et al. (11).
[d] Data from Yabuuchi et al. (35).
[e] Data from Mudarris et al. (24).
[f] Mean ± standard deviation.
[g] Number of strains positive/number of strains tested.
[h] V, variable. The number of strains positive is not available.
[i] NA, not available.

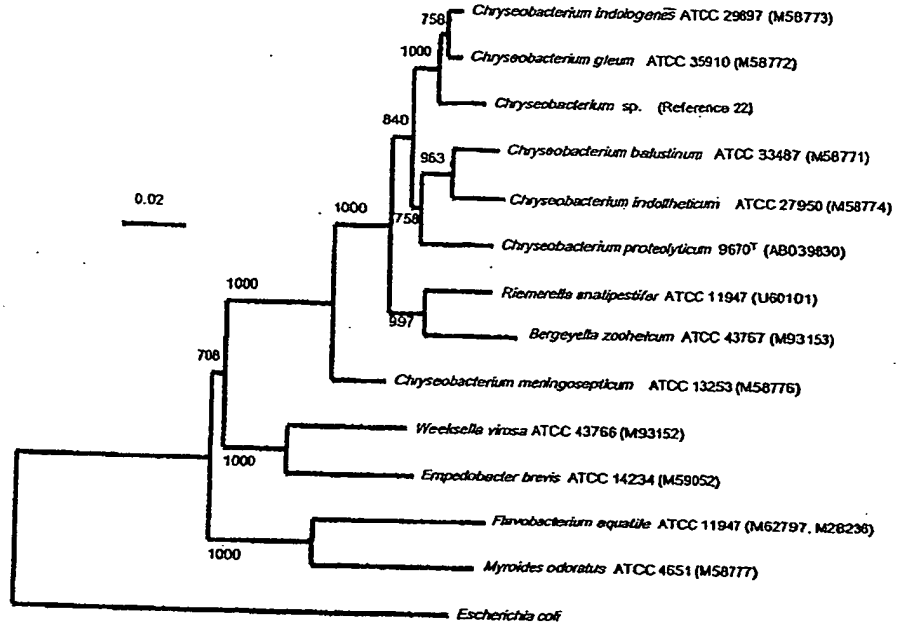

FIG. 5. Phylogenetic position of strain 9670^T within the genus *Chryseobacterium* and the allied bacteria. The branching pattern was generated by the neighbor-joining method. The accession numbers of the 16S rRNA nucleotide sequences used are indicated in parentheses. The 16S rRNA sequence of *Chryseobacterium* sp. was obtained from reference 22. The number at each branch indicates the bootstrap values. Bar, 0.02 nucleotide substitutions per site.

of *Riemerella anatipestifer*, *Bergerella zoohelicom*, and *C. meningosepticum*, with 92.2 to 93.5% similarities. Other related strains, such as *Weeksella virosa*, *Empedobacter brevis*, *Flavobacterium aquatile*, and *Myroides odoratus*, had 83.6 to 87.1% similarities. A phylogenetic tree constructed by the neighbor-joining method showed that strain 9670^T exists as an independent branch within the above-mentioned group having higher sequence similarities (Fig. 5). The bootstrap analysis resulted in relatively high values of more than 75% for all of the branches within this group.

DNA base composition and DNA-DNA hybridization. The G+C content of strains 9670^T and 9671 was 37.1 mol%. The levels of DNA-DNA relatedness were estimated by using these two strains, four type strains from *Chryseobacterium* (*C. balustinum*, *C. gleum*, *C. indologenes*, and *C. meningosepticum*), and two other related type strains (*Empedobacter brevis* and *Myroides odoratus*) (Table 2). The DNA-DNA relatedness value between strains 9670^T and 9671 was 94%. Low values (14 to 17%) of relatedness were observed between the new isolates and three strains, *C. gleum*, *C. indologenes*, and *C. balustinum*, which showed higher similarities in 16S rRNA gene sequences to strain 9760^T. The values for strain 9670^T to three other strains, *C. meningosepticum*, *E. brevis*, and *F. odoratus*, were only 8 or 7, 4 and 3%, respectively. A relatively higher value (31%) in this analysis was observed between *C. gleum* and *C. indologenes*. All of these results supported the phylogenetic tree illustrated from 16S rRNA gene sequence analysis (Fig. 5).

DISCUSSION

Protein-deamidating activity was found in the culture supernatant of a newly isolated bacterium, strain 9670^T. Both Cbz-Gln-Gly- and casein-deamidating activities and protease activ-

TABLE 2. Levels of DNA-DNA relatedness between strains 9670^T and 9671 and various test isolates

Strain[a]	% DNA-DNA relatedness to:							
	IFO 12535^T	JCM 2410^T	IFO 15053^T	IFO 14944^T	IFO 14943^T	IFO 14945^T	9670^T	9671
C. meningosepticum IFO 12535^T	100							
C. gleum JCM 2410^T	10	100						
C. balustinum IFO 15053^T	8	14	100					
C. indologenes IFO 14944^T	9	31	14	100				
E. brevis IFO 14943^T	7	7	8	8	100			
M. odoratus IFO 14945^T	5	4	4	4	5	100		
Strain 9670^T	8	17	14	17	4	3	100	
Strain 9671	7	17	NT[b]	NT	NT	NT	94	100

[a] IFO, Institute for Fermentation, Osaka, Japan; JCM, Japan Collection of Microorganisms, Wako, Japan.
[b] NT, not tested.

ity were produced synchronously during the course of culture. The deamidating enzyme was separated from proteases by phenyl-Sepharose chromatography. Native caseins were not degraded by the enzyme, while more than 60% of amido groups in caseins were estimated to be deamidated, indicating that the enzyme can deamidate high-molecular-weight peptides (i.e., proteins) in the native state. The enzyme from strain 9670^T is the first protein-deamidating enzyme of microbial origin. From the industrial point of view, the microbial protein-deamidating enzyme has great significance, because it opens the way to mass production of such an enzyme, which will find many applications.

Two peptidoglutaminases that catalyze the deamidation of peptide-bound glutamine residues have been found in *Bacillus circulans*: peptidoglutaminase I (EC 3.5.1.43), which deamidates the γ-carboxyamido group of C-terminal glutamine residue; and peptidoglutaminase II (EC 3.5.1.44), which deamidates the γ-carboxyamido groups of N-terminal and internal glutaminyl residues. Both enzymes, however, cannot deamidate caseins unless the caseins are prehydrolyzed (17). Gill et al. (8) reported that peptidoglutaminases are not active against caseins and whey proteins even after denaturation and are only active against glutaminyl residues in peptides with a molecular weight below 5,000. Hamada (9) reported slight enhancements of peptidoglutaminase-catalyzed protein deamidation using heat- and/or alkaline-treated proteins, but the degrees of deamidation were very low (0.8 to 3.0% for caseins) compared to those for the preparations treated by the combinations with proteolysis (ca. 38%).

Transglutaminase (EC 2.3.2.13) is an enzyme with a wide distribution ranging from mammals to microorganisms. The enzyme catalyzes the acyl transfer reaction in which the γ-carboxyamido groups of glutaminyl residue in proteins or peptides are the acyl donor. A variety of amines, including lysyl residues of proteins, can act as acyl acceptors. When lysyl residues of protein act as acyl acceptors, cross-linked products with higher molecular weights are formed through intermolecular isopeptide bonding. In the absence of amines in the reaction system, water can act as an acyl acceptor, resulting in the deamidation of glutaminyl residues in proteins. The protein-deamidating enzyme from strain 9670^T was distinguished from transglutaminase, because no transglutaminase activities were detected based on the lack of hydroxyamate formation and lack of cross-linked product formation from caseins.

The physiological role of the protein-deamidating enzyme produced by the microorganism is unknown. In germinating seeds in plants, deamidations of storage proteins preceeding their proteolytic degradation were observed, and the possible involvement of a protein-deamidating enzyme in this process has been pointed out (31). Observed simultaneous expressions of the protein-deamidating enzyme and proteases into a culture medium by strain 9670^T (Fig. 1) may suggest the involvement of a protein-deamidating enzyme in the degradation process of proteins to be utilized as energy or nutritional sources in cooperation with proteases. It was reported that proteins isolated from germinated seed, which were deamidated and conformationally changed, had an increased susceptibility to proteolytic digestion (19).

Two strains, 9670^T and 9671, were isolated from soils in natural environments of the Tsukuba area, Japan, as producers of the protein-deamidating enzyme. Phenotypic characterization, 16S rRNA sequencing, and DNA-DNA hybridization studies indicated the isolates belonged to a new species in the genus *Chryseobacterium*. The genus *Chryseobacterium* is an emended one for some strains originally isolated as "*Flavobacterium*-like bacteria." In 1994, Vandamme et al. (33) proposed that six strains (*F. gleum*, *F. indologenes*, *F. balustinum*, *F. indoltheticum*, *F. meningosepticum*, and *F. scophthalmum*) should be given a new separate status based on the previously reported phenotypic and chemotaxonomic features as well as rRNA cluster analysis, and they coined a new name, *Chryseobacterium*, for these strains. The well-characterized species *C. gleum* (11) was selected as the type species of this genus. They pointed out that *C. meningosepticum*, well known as a pathogenic strain, had the most aberrant features within this genus. In this study, we recognized the newly isolated strains should be placed in the genus *Chryseobacterium* based on the results from both phenotypic and DNA analyses (DNA-DNA hybridization and 16S rRNA sequencing). DNA analyses also indicated that the isolates were closely related to a group of *Chryseobacterium* species, except for *C. meningosepticum*.

The strains belonging to *Chryseobacterium* have been isolated from various ecosystems, such as water, soils, fish, marine environments, and clinical specimens. Many bacteria isolated from food environments, such as milk and butter (15), were recently recognized as members of the genus *Chryseobacterium* (12, 13). More recently, an isolate from fish was determined as a strain belonging to the genus by 16S rRNA analysis (22). The isolates we studied here were from soils of a rice field and the bank of a brook in Japan. These recent reports suggest a wide distribution of *Chryseobacterium* strains in various natural environments, although early studies of the taxonomy of "*Flavobacterium*-like bacteria," some of which are presently placed in *Chryseobacterium*, had been mainly focused on clinical strains. The new isolates reported here produced highly proteolytic activities. This characteristic was also mentioned for some *Chryseobacterium* strains (originally isolated as flavobacteria) from dairy foods (14).

We propose a new species with the name *Chryseobacterium proteolyticum* sp. nov. Strain 9670 was designated the type strain of *Chryseobacterium proteolyticum*. A description of the new species is given below.

Description of *Chryseobacterium proteolyticum* sp. nov. *Chryseobacterium proteolyticum* (pro.te.o.ly'ti.cum. Gr. n. *proteo*; Gr. adj. *lyticus*, dissolving; M.L. neut. adj. *proteolyticum*, protein dissolving, proteolytic). Cells are gram-negative, nonsporeforming, and nonmotile rods (0.4 to 0.5 by 0.8 to 2.0 μm). Circular, orange or light-pinkish cream colonies are formed on nutrient agar at 30°C for 2 days. Yellow or orange insoluble, flexirubin-type pigment is produced. The organism shows growth at 37°C but not at 42°C. The pH range for growth is from 5 to 9 and that for optimal growth is 6 to 8. Growth occurs aerobically, not anaerobically. No growth is observed on MacConkey agar. Catalase and cytochrome oxidase reactions are positive, but urease negative. The organism is positive for indole production, weakly positive for H_2S formation, but negative for malonate utilization, nitrate reduction, denitrification, 3-ketolactose formation, and the Voges-Proskauer test. Lysine decarboxylase, arginine dihydrase, ornithine decarboxylase, and phenylalanine deaminase are negative. Hydrolyses of esculin, Tween 80, starch, tyrosine, casein, gelatin, DNA, and *o*-nitrophenyl-β-D-galactopyranoside are positive. The organism produces acid from L-arabinose, D-glucose, maltose, sucrose, trehalose, and soluble starch; produces acid weakly from glycerol and mannitol; and does not produce acid from adonitol, cellobiose, ethanol, inositol, inulin, lactose, raffinose, rhamnose, or salicin. The G + C content of the DNA is 37.1 mol% (determined by high-performance liquid chromatography). Strains 9670^T and 9671 were obtained from soil samples from Tsukuba, Ibaraki, Japan. The type strain, 9670, has been deposited in the Patent Microorganism Depository, National Institute of Bioscience and Human Technology (Tsukuba, Ja-

pan), as strain FERM P-17664. The strain will be made available for research.

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